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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/00, 5/02

A1

(11) International Publication Number: WO 97/40137

(43) International Publication Date: 30 October 1997 (30.10.97)

(21) International Application Number: PCT/US97/06433

(22) International Filing Date: 17 April 1997 (17.04.97)

(30) Priority Data:

60/016,245	19 April 1996 (19.04.96)	US
60/029,838	28 October 1996 (28.10.96)	US

(60) Parent Applications or Grants

(63) Related by Continuation

US	60/016,245 (CIP)
Filed on	19 April 1996 (19.04.96)
US	60/029,838 (CIP)
Filed on	28 October 1996 (28.10.96)

(71) Applicant *(for all designated States except US)*: OSIRIS THERAPEUTICS, INC. [US/US]; 2001 Aliceanna Street, Baltimore, MD 21231-2001 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: KADIYALA, Sudha [IN/US]; 1531 Lancaster Street, Baltimore, MD 21231 (US). BRUDER, Scott, P. [US/US]; 3698 Ashley Way, Owings Mills, MD 21117 (US). MUSCHLER, George, F. [US/US]; 2270 Chatfield Road, Cleveland Heights, OH 44106 (US).

(74) Agents: HERRON, Charles, J. et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

With international search report.

(54) Title: REGENERATION AND AUGMENTATION OF BONE USING MESENCHYMAL STEM CELLS

(57) Abstract

Disclosed are compositions and methods for augmenting bone formation by administering isolated human mesenchymal stem cells (hMSCs) with a ceramic material or matrix or by administering hMSCs; fresh, whole marrow; or combinations thereof in a resorbable biopolymer which supports their differentiation into the osteogenic lineage. Contemplated is the delivery of (i) isolated, culture-expanded, human mesenchymal stem cells; (ii) freshly aspirated bone marrow; or (iii) their combination in a carrier material or matrix to provide for improved bone fusion area and fusion mass, when compared to the matrix alone. The material or matrix can be a granular ceramic or three-dimensionally formed ceramic implant. The material or matrix can also be a resorbable biopolymer. The resorbable biopolymer is an absorbable gelatin, colagen or cellulose matrix, can be in the form of a powder or sponge, and is preferably a bovine skin-derived gelatin. The implants can be shaped as a cube, cylinder, block or an anatomical site. The compositions and methods can further include administering a bioactive factor such as a synthetic glucocorticoid, like dexamethasone, or a bone morphogenic protein, like BMP-2, BMP-3, BMP-4, BMP-6 and BMP-7.

REGENERATION AND AUGMENTATION OF BONE USING MESENCHYMAL STEM CELLS

This application is a continuation-in-part of U.S. provisional application serial no. 60/016,245, filed April 19, 1996 and U.S. provisional application serial no. 60/029,838 filed October 28, 1996.

Autologous, culture-expanded, bone marrow-derived MSCs have now been shown to regenerate clinically significant bone defects. Using techniques for isolating and cultivating human MSCs, it should be possible to implement therapeutic strategies based on the administration of a patient's own cells which have been harvested by a simple iliac crest aspiration. This method may provide an alternative to autogenous bone grafting, and will be particularly useful in clinical settings such as ageing and osteoporosis, where the number and/or function of endogenous MSCs have been reduced.

The repair of large segmental defects in diaphyseal bone is a significant problem faced by orthopaedic surgeons. Although such bone loss may occur as the result of acute injury, these massive defects commonly present secondary to congenital malformations, benign and malignant tumors, osseous infection, and fracture non-union. The use of fresh autologous bone graft material has been viewed

Summary of the Invention

The present invention provides compositions and methods for directing MSCs cultivated *in vitro* to differentiate into specific cell lineage pathways prior to, and/or at the time of, their implantation for the therapeutic treatment of elective procedures or pathologic conditions in humans and other species. The use of both autologous and allogenic MSCs is contemplated in this invention.

The investigations reported here confirm the *in vitro* and *in vivo* osteogenic potential of MSCs; demonstrate the *in vivo* osteogenic potential of MSCs when implanted at an ectopic subcutaneous site; and illustrate that purified, culture-expanded MSCs can regenerate a segmental bone defect which would otherwise result in a clinical non-union. These experiments compared the healing potential of MSCs delivered in an osteoconductive, osteoinductive or other appropriate resorbable medium. We also show *de novo* formation of bone at the site of a desired fusion, *e.g.* spinal or joint fusions.

The invention provides a method for augmenting bone formation in an individual in need thereof by administering isolated human mesenchymal stem cells with a matrix which supports the differentiation of such stem cells into the osteogenic lineage to an extent sufficient to generate bone formation therefrom. The matrix is preferably selected from a ceramic and a resorbable biopolymer. The ceramic can be in particulate form or can be in the form of a structurally stable, three dimensional implant. The structurally stable, three dimensional implant can be, for example, a cube, cylinder, block or an appropriate anatomical form. The resorbable biopolymer is a gelatin, collagen or cellulose matrix, can be in the form of a powder or sponge, and is preferably a bovine skin-derived gelatin.

Particularly, the invention provides a method for effecting the repair or regeneration of bone defects in an animal or individual in need thereof. Such defects include, for example, segmental bone defects, non-unions, malunions or delayed unions, cysts, tumors, necroses or developmental abnormalities. Other conditions requiring bone augmentation, such as joint reconstruction, cosmetic reconstruction or bone fusion, such as spinal fusion or joint fusion, are treated in an individual by

More particularly, the invention provides a method for effecting the repair of segmental bone defects, non-unions, malunions or delayed unions in an individual in need thereof by administering into the bone defect of said person isolated human mesenchymal stem cells in a porous ceramic carrier, thereby inducing the differentiation of such stem cells into the osteogenic lineage to an extent sufficient to generate bone formation therefrom. Preferably, the porous ceramic carrier comprises hydroxyapatite and, more preferably, the porous ceramic carrier further comprises β-tricalcium phosphate. The porous ceramic carrier may also contain one or more other biodegradable carrier components which degrade, resorb or remodel at rates approximating the formation of new tissue extracellular matrix or normal bone turnover.

The invention also provides for the use of other extracellular matrix components, or other constituents, so as to achieve osteoconductive or osteoinductive properties similar to natural extracellular matrix. The composition is an absorbable gelatin, cellulose and/or collagen-based matrix in combination with bone marrow and/or isolated mesenchymal stem cells. The composition can be used in the form of a sponge, strip, powder, gel, web or other physical format. The composition is, for example, inserted in the defect and results in osteogenic healing of the defect.

In addition, by varying the ratios of the components in said biodegradable matrices, surgical handling properties of the cell-biomatrix implants can be adjusted in a range from a porous ceramic block or a moldable, putty-like consistency to a pliable gel or slurry.

More particularly, the invention comprises a rigid cell-matrix implant for large segmental defects, spinal fusions or non-unions, gel or slurry cell-matrix implants, or infusions for stabilized fractures and other segmental bone defects. Custom cell-matrix implants containing autologous or allogeneic MSCs can be administered using open or arthroscopic surgical techniques or percutaneous insertion, *e.g.* direct injection, cannulation or catheterization.

material may be uncoated, or coated with a variety of materials including autologous serum, purified fibronectin, purified laminin, or other molecules that support cell adhesion. The granular ceramic material can be combined with MSCs ranging in concentration from 10 thousand to 30 million cells per cc of ceramic, with a preferred range between 3 and 15 million cells per cc. It is also envisioned that the cells may be in the form of fresh marrow obtained intraoperatively, without *ex vivo* culture-expansion.

Bone marrow cells may be obtained from iliac crest, femora, tibiae, spine, rib or other medullary spaces. Other sources of human mesenchymal stem cells include embryonic yolk sac, placenta, umbilical cord, periosteum, fetal and adolescent skin, and blood. The cells are incubated at 37°C with the ceramic for 0 to 5 hours, preferably 3 hours. Prior to implant, the cell-loaded granules can be combined with either fresh peripheral blood, human fibrin, fresh bone marrow, obtained by routine aspiration, or other biological adjuvant. These final combinations are allowed to form a soft blood clot which helps to keep the material together at the graft site. Implant or delivery methods include open or arthroscopic surgery and direct implant by injection, *e.g.* syringe or cannula. Finally, these implants may be used in the presence or absence of fixation devices, which themselves may be internally or externally placed and secured.

The composition can also contain additional components, such as osteoinductive factors. Such osteoinductive factors include, for example, dexamethasone, ascorbic acid-2-phosphate, β-glycerophosphate and TGF superfamily proteins, such as the bone morphogenic proteins (BMPs). The composition can also contain antibiotic, antimycotic, antiinflammatory, immunosuppressive and other types of therapeutic, preservative and excipient agents.

each group. The radiodensity of the HA/TCP material reveals the porous nature and the central canal of each implant.

Figures 3A and 3B. Defects left empty;

Figures 3C and 3D. Defects fitted with HA/TCP carrier alone;

Figures 3E and 3F. Defects fitted with a MSC-loaded HA/TCP carrier;

Figures 3G and 3H. Defects fitted with a marrow-loaded HA/TCP carrier. Defects left empty following segmental gap resection undergo reactive bone formation at the cut ends of the bone, leading to a classical non-union in this well established model. At four weeks, the MSC-loaded samples have begun to fill the pores of the implant material. No union is evident in any implant type at four weeks. By eight weeks, modest union of the host-implant interface has occurred in the carrier (d) and carrier plus marrow groups (h), but complete integration and bone bridging is evident in the carrier plus MSC group (f). Total filling of the pores with bone in the MSC-loaded sample is also evident in panel *F*. (x1.5)

Figure 4A-4F. Light micrographs showing representative healing of the segmental defect at four and eight weeks with various implant types. Intact limbs were harvested, fixed, dehydrated, cleared, embedded in polymethylmethacrylate, cut, and ground to 100 micron thickness prior to staining. Some animals received India ink injections to allow visualization of the vascular tree, present here in panels *B*, *C*, *D*, and *E* as black staining. The HA/TCP material artifactually appears black in these photomicrographs as a result of undecalcified processing. The cut edges of the host cortices are noted by arrowheads in *a* and *b*, and similar sections are presented in all other panels.

Figures 4A and 4B. Defects fitted with HA/TCP carrier alone at four and eight weeks, respectively;

Figure 6C. APase activity and calcium deposition in MSC cultures grown in Control or OS Medium on days 4, 8, 12 and 16. Samples were harvested at the indicated days, and APase activity, cell number, and calcium deposition were determined as described in Materials and Methods. The results represent the mean ± SD of triplicate cultures from first passage cells. $*P<0.05$, $\dagger P<0.005$ (compared to Control).

Figure 7. Light micrograph of a representative histological section from a human MSC-loaded HA/TCP implant placed ectopically in subcutaneous tissue of an athymic rat. MSCs were loaded into the ceramic, implanted as described in Materials and Methods, harvested at 12 weeks, decalcified and processed in paraffin for microscopy. Only remnants of the HA/TCP ceramic (c) remain, while the pores of the implant are filled with bone (b), blood vessels (arrow) or fibrous tissue (f). Cuboidal osteoblasts are seen lining the surface of the developing bone. (Toluidine blue-O, x75).

Figure 8. Segmental gap defect model and radiography. (*a*) A polyethylene fixation plate is positioned on the lateral aspect of this representative rat femur. Four bicortical screws and 2 cerclage wires are used to secure the plate in place. An 8 mm segment of bone is removed along with its adherent periosteum, and a ceramic implant, with or without cells, is placed into the defect site. The overlying muscles are returned to their proper anatomic position, and the skin is closed with resorbable sutures. High resolution radiographs obtained immediately following sacrifice show the extent of healing of the segmental defect at 12 weeks with the 2 implant types (*b*, *c*). While total integration of the implant at the host-ceramic interface is evident in the carrier plus MSC group (*b*), only modest union is observed in the cell-free implants (*c*). The pores of the MSC-loaded implant are filled with bone throughout the gap, but the cell-free carrier contains little bone and several cracks.

Figure 9. Histologic representation of bone regeneration in segmental femoral defects. Immunohistochemical staining with antibody 6E2 (*a*) demonstrates that 4 weeks following implantation of a MSC-loaded sample, the

Figure 11. The grid system used to assign union score is shown in schematic cross-section at the level of the facet joints. One half point is assigned for union in each of the box areas.

Detailed Description of the Invention

Bone grafting procedures are widely used to treat acute fractures, fracture non-unions, bone defects, and to achieve therapeutic arthrodesis. Autogenous cancellous bone is the current "gold standard" for clinical bone grafting. Contemporary dogma attributes this effectiveness to three primary intrinsic properties: osteoconduction, osteogenic cells, and osteoinduction (76,96), which can be defined as follows:

Osteoconduction - The scaffold function provided by the transplanted extracellular bone matrix which facilitates cell attachment and migration, and therefore the distribution of a bone healing response throughout the grafted volume. This property is likely dependent on adhesion molecules within bone matrix such as: collagens, fibronectin, vitronectin, osteonectin, osteopontin, osteocalcin, proteoglycans and others. Growth factors in the matrix may also play a role.

Osteogenic cells - Those cells in the autograft derived from bone or bone marrow which survive transplantation and go on to proliferate and/or undergo osteoblastic differentiation.

Osteoinduction - The bioactive property of autogenous bone derived from the presence of growth factors or other elements in the graft which stimulate the proliferation and/or differentiation of osteoblastic progenitors. Many growth factors have been identified in bone matrix including: bone morphogenetic proteins (BMPs), transforming growth factor-beta (TGF-β), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF). Transplanted non-osteogenic cells in bone marrow may also elaborate factors which contribute

The investigations reported here confirm the *in vivo* healing potential of fresh whole marrow or MSCs delivered in the matrix alone, or in the matrix in combination.

The invention also contemplates the use of other extracellular matrix components, along with the cells , so as to achieve osteoconductive or osteoinductive properties. In addition, by varying the ratios of the components in said biodegradable matrices, surgical handling properties of the cell-biomatrix implants can be adjusted in a range from a dimensionally stable matrix, such as a sponge or film, to a moldable, putty-like consistency to a pliable gel or slurry to a powder.

The marrow or isolated mesenchymal stem cells can be autologous, allogeneic or from xenogeneic sources, and can be embryonic or from post-natal sources. Bone marrow cells may be obtained from iliac crest, femora, tibiae, spine, rib or other medullary spaces. Other sources of human mesenchymal stem cells include embryonic yolk sac, placenta, umbilical cord, periosteum, fetal and adolescent skin, and blood. In order to obtain mesenchymal stem cells, it is necessary to isolate rare pluripotent mesenchymal stem cells from other cells in the bone marrow or other MSC source.

In a particularly preferred embodiment, the composition of the invention comprises an absorbable implant, containing whole marrow and/or isolated MSCs for repair of segmental defects, spinal fusions or non-unions and other bone defects. Custom cell-matrix implants containing autologous, allogeneic or xenogeneic bone marrow and/or MSCs can be administered using open surgical techniques, arthroscopic techniques or percutaneous injection.

Human mesenchymal stem cells (hMSCs) can be provided as either homogeneous, culture-expanded preparations derived from whole-marrow (or other pre-natal or post-natal source of autologous or allogeneic hMSCs), from hMSC-enriched or heterogenous cultures or fresh, whole marrow (when combined with an osteoinductive or other optimized medium) containing an effective dose of

In the embodiment which uses a cellulose-based matrix, an appropriate absorbable cellulose is regenerated oxidized cellulose sheet material, for example, Surgicel® (Johnson & Johnson, New Brunswick, NJ.) which is available in the form of various sized strips or Oxycel® (Becton Dickinson, Franklin Lakes, NJ) which is available in the form of various sized pads, pledgets and strips. The absorbable cellulose-based matrix can be combined with the bone reparative cells and, optionally, other active ingredients by soaking the absorbable cellulose-based matrix in a cell suspension of the bone marrow and/or MSC cells, where the suspension liquid can have other active ingredients dissolved therein. Alternately, a predetermined amount of a cell suspension can be transferred on top of the cellulose-based matrix, and the cell suspension can be absorbed.

In the embodiment which uses a collagen-based matrix, an appropriate resorbable collagen is purified bovine corium collagen, for example, Avitene® (MedChem, Woburn, MA) which is available in various sizes of nonwoven web and fibrous foam, Helistat® (Marion Merrell Dow, Kansas City, MO) which is available in various size sponges or Hemotene® (Astra, Westborough, MA) which is available in powder form. The resorbable collagen-based matrix can be combined with the bone reparative cells and, optionally, other active ingredients by soaking the resorbable collagen-based matrix in a cell suspension of the bone marrow and/or MSC cells, where the suspension liquid can have other active ingredients dissolved therein. Alternately, a predetermined amount of a cell suspension can be transferred on top of the collagen-based matrix, and the cell suspension can be absorbed.

The above gelatin-based, cellulose-based and collagen-based matrices may, optionally, possess hemostatic properties.

Preferred active ingredients are those biological agents which enhance wound healing or regeneration of bone, particularly recombinant proteins. Such active ingredients are present in an amount sufficient to enhance healing of a wound, *i.e.*, a wound healing-effective amount. The actual amount of the active ingredient will be determined by the attending clinician and will depend on

CO_2. Non-adherent cells were removed at the time of the first medium change, four days post plating, and cells were routinely fed twice weekly thereafter. These primary cultures approached confluence typically at thirteen days, were then released by a five minute exposure to 0.25% trypsin containing one millimolar EDTA, and subcultivated at a density of 10^4 cells/cm^2. Cells for implantation were derived from these first passage cultures ten days after replating, at which time they were approximately 85% confluent.

***In Vitro* Osteogenic Assays**

At the end of first passage, MSCs were replated into six-well plates at a density of 10^4 cells/cm^2 in Control Medium. The following day (Day 0), fresh Control Medium was provided, and the cells were grown in the absence or presence of Osteogenic Supplements (OS) (100 nanomolar Dex, 0.05 millimolar AsAP and ten millimolar β-GP) (64). Media changes were performed twice weekly, and at days seven, fourteen, twenty-one, and twenty-eight, cultures were assayed for cell number, alkaline phosphatase (APase) histochemistry, and mineralized matrix production utilizing techniques previously described (64).

Implant Preparation

HA/TCP blocks were shaped into cylinders approximately four millimeter in diameter and eight millimeter in length. A central canal roughly one millimeter in diameter was bored through the length of the entire cylinder using an eighteen gauge hypodermic needle. Cylinders were cleaned by sonication and rinsing in distilled water, and then sterilized by 220°C dry heat for five hours. The cylinders were subsequently coated with human plasma fibronectin (Cal-Biochem, Irvine, CA) by soaking in a 100 microgram per milliliter solution for sixteen hours at 4°C. The implants were then air dried at room temperature overnight in a sterile biosafety cabinet, and stored at 4 °C. HA/TCP cubes, measuring three millimeter per side, were similarly prepared and coated with fibronectin as described above for use in the ectopic osteogenesis assay.

HA/TCP implants, both in cube and cylinder form, were loaded with MSCs using a modification of a technique previously described (32,83). Briefly,

implants of the MSC-loaded HA/TCP cubes to correlate the ectopic osteogenesis assay with orthotopic bone regeneration and the in vitro osteogenic potential of syngeneic MSCs. Rats implanted with marrow-loaded cylinders similarly received subcutaneous implants of marrow-loaded cubes. The animals were allowed full activity in their cages post-operatively. No animals experienced failure of fixation or other post-operative complications. At least six limbs were used for each of the implant groups, randomly selected between left or right. Upon sacrifice at four and eight weeks, the vascular tree of some animals was perfused with India ink, and the entire femur and surrounding soft tissue was carefully dissected. Specimens were immediately evaluated radiographically, and subsequently processed for undecalcified histology.

Radiographic Analysis

The specimens were radiographed using a high resolution Faxitron Imaging system (Buffalo Grove, IL) with an exposure of thirty-five kVP for thirty seconds. The radiographs were independently evaluated by two of the authors who were blinded with respect to the duration and type of implant. Bone formation was scored on a semiquantitative scale with ranges as follows: distal host-implant union (0-2); proximal host-implant union (0-2); and implant core density (0-4). The union scores and the core density scores were added to give a maximum possible score of eight for each implant. Results from both examiners were averaged to give final scores.

Histology and Histomorphometry

Following fixation in 10% buffered formalin, the femurs were dehydrated, cleared, and embedded in polymethylmethacrylate. Longitudinal sections were cut on a water-cooled Isomet saw (Buehler, WI), and a central section of each leg was ground to 100 micrometer thickness, polished, and stained with Toluidine blue-O. Leica Quantimet 500MC (Cambridge, UK) image analysis software was used to determine the area of HA/TCP implant, bone, and soft tissue in the diaphyseal defect region of each section. The data were analyzed by one-way analysis of variance (ANOVA) (Sigmastat, Jandel Scientific). Further analyses were performed according to *post hoc* Student-Newman-Keuls tests.

MSC-Mediated Osteogenesis in Ectopic HA/TCP Implants

All MSC-loaded HA/TCP cubes implanted in the host rats had ample evidence of osteogenesis by four weeks. At the eight week time point, a substantial amount of bone, and occasionally cartilage, was present within the pores of the cubes. A representative section from a MSC-loaded cube harvested eight weeks following implantation is shown in Figure 2. The unstained granular areas reflect the former regions of ceramic material which have been removed during the decalcification step of specimen preparation. As seen in the photomicrograph, bone formation occurs within the pores of the cubes, and is associated with vascular elements which penetrate the implant. Such angiogenesis is obligatory to new bone formation since the secretory activity of osteoblasts is an oriented phenomenon guided by vasculature. Both woven and lamellar bone can be seen depending on the duration of implantation, and the precise region examined. Most of the pores are filled with bone and small islands of hematopoietic elements, with the remainder being filled with a loose connective tissue. In contrast to these MSC-loaded samples, cubes loaded with fresh marrow contained negligible osseous tissue at four weeks, and only slightly more even at eight weeks. As previously demonstrated (32,54), cubes implanted without MSCs or marrow contained no bone, but were filled with fibrous tissue and blood vessels.

Radiographic Evaluation

High resolution Faxitron radiographs provided sufficient clarity and detail to discern subtle changes occurring within the implant and the surrounding host bone. Figure 3 shows representative radiographs of the femurs from each of the groups recovered at four and eight weeks post-implantation. As demonstrated in these radiographs, the fixation remained intact in all the samples and there were no fractures in any of the femurs. In animals whose femoral defects were left empty, reactive bone formation at the transversely cut edges of the host femur was observed at four weeks (Fig. 3A). By eight weeks, slightly more bone was present within the gap, however, most of this bone appeared to form along the edge of the fixation plate which was in contact with the periosteum (Fig. 3B). Every specimen which was left empty resulted in the formation of a radiographic

Table 1

Average of Radiographic Scores for Each Implant Group

	Four Weeks			Eight Weeks		
	C only	C+MSCs	C+M	C only	C+MSCs	C+M
Proximal Union	1.5	0.8	0.2	1.2	1.3	1.0
Distal Union	0.5	1.4	0.7	1.2	2.0	1.6
Core Density	0.7	2.0	0.3	0.6	3.7	0.7
Total Score	2.7	4.2	1.2	3.0	7.0*	3.3

Table 1. Average of radiographic scores for each implant group at each time point. C = carrier, M = marrow. Radiographs were evaluated and scored by two independent observers blinded to the identity of each implant. Union was scored both proximally and distally on a scale of 0-2. Core density was scored on a scale of 0-4. n=3 for each group at each time point. The maximum possible total score is 8. One-way analysis of variance at the two different time points, with cell loading (none, MSCs, and marrow) as the independent variable showed significant difference between groups at 8 weeks ($F = 10.9$, $p = 0.01$) but were not significantly different at four weeks. * = significantly greater ($p < 0.05$) than other groups at the corresponding time point (according to *post hoc* Student-Newman-Keuls tests).

In the case of the defects filled with the HA/TCP carrier alone, the low scores indicate the absence of any radiodense material within the pores, and minimal union of the implant with the host bone. Loading the HA/TCP implant with fresh marrow did not result in an improvement in the healing of the defect, and the low scores reflect the similarity of this group to that of the carrier alone. However, loading the HA/TCP carrier with MSCs produces a vigorous osteogenic response. Even at four weeks, pore filling was observed and is reflected in the considerably higher scores of these implants. Interestingly, even in this case the host-implant union was modest compared to controls. By the eight week time point, the pores of the implant were filled with new bone and the host-implant union was well established.

highly cellular, and is presented in higher magnification photomicrographs in Figure 5. New woven and lamellar bone can be seen in intimate contact with the cut edge of the host cortex at eight weeks (Fig. 5A). Importantly, this region of union is directly contiguous with bone formed throughout the pores of implant. In regions deeper within the HA/TCP, filling of the pores with new bone is evident, as is the association of vasculature which orients the secretory activity of the differentiating osteoblasts (Fig. 5B).

The results qualitatively described above are mirrored in the histomorphometric data presented in Table 2.

Table 2

Carrier alone	Carrier + MSCs	Carrier + Marrow
2.3 ± 1.5	19.3 ± 3.7*	2.9 ± 1.7
10.4 ± 2.4	43.3 ± 7.7*	17.2 ± 6.0

Table 2. Bone fill in HA/TCP implants as a percentage of available space. Histomorphometric measurements were obtained on the bone formed within the confines of the segmental resection, excluding the implant material itself and the medullary canal. The values are reported as means of three samples along with standard deviations from the mean. One-way analysis of variance at the two different time points, with cell loading (none, MSCs, and marrow) as the independent variable showed significant difference between MSC-loaded samples at both 4 weeks ($F = 43.3$, $p < 0.001$) and 8 weeks ($F = 26.2$, $p < 0.002$). * = significantly greater ($p < 0.01$) than other groups at the corresponding time points (according to *post hoc* Student-Newman-Keuls tests). No difference was observed between marrow and carrier alone at either time point ($p > 0.1$).

The cell-free HA/TCP implants had a bone fraction of only 2.3% and 10.4% at four and eight weeks, respectively. Importantly, this fraction of bone at eight weeks correlates with previously published results (126). These fractions primarily represent the bone ingrowth from the cut ends of the host cortices. The marrow-loaded HA/TCP cylinders did exhibit modest osteogenesis within the

To further characterize the cells used in this study, we cultured them in the presence and absence of a medium which induces osteogenic differentiation *in vitro*. As has been reported in numerous other laboratories (77,88,89,118,135), these rat marrow-derived cells develop along the osteogenic lineage in response to dexamethasone, eventually forming mineralized nodules of bone-like tissue on the surface of the dish. Such differentiation is evident in our photomicrographs (Fig. 1), and serves to document that the cells used in these implants indeed possess the ability to form bone, one of the inherent properties of MSCs. Furthermore, the bone and cartilage formed in cubes implanted subcutaneously not only confirms the osteochondral potential of the MSCs, but acts as an internal control to verify that every host rat was capable of providing an environment which could support osteogenesis within these combined cell:matrix implants. Additional experiments documenting the multilineage potential of these cells were not included as part of the current study because previous publications have focused on describing such potential in greater detail (32,79,80,121,143). The isolation and selection procedures for rat MSCs are similar to those used for human MSCs (32,54,80), and result in the formation of characteristic primary colonies illustrated in Figure 1A. These cells are mitotically expanded to yield a morphologically homogeneous population which divides uniformly across the dish. Both human and rat MSCs have been shown to possess multilineage potential, and the details of *in vitro* osteogenic differentiation of human MSCs has recently been reported (33,64). Conditions for the isolation and culture expansion of human MSCs without lineage progression have been optimized (13,54,80), and the development of a serum free medium for human MSC growth has been completed (58).

The radiographic findings in this study establish a precedent for obtaining non-invasive evidence of bone regeneration in animals, or humans, which receive MSCs in an orthotopic location. Given the porous nature of the HA/TCP implants, new bone which forms within the interstices of the material is readily apparent radiographically by four weeks, in spite of the inherent radiodensity of the HA/TCP material. The progressive increase in radiodensity evident by eight weeks correlates well with the histological observations of processed limbs. Interestingly, despite the presence of new bone within the core of implants by

The ability of MSCs to regenerate a large segmental defect in this experimental model compares favorably with other investigations testing implants such as demineralized bone matrix, bone marrow, purified or recombinant BMPs, allograft, ceramics, and fibermetals (34,37,74,83,105,126,129,144,145,147). While the use of recombinant BMP has received considerable attention, the precise mechanism of action has only recently been appreciated. These powerful inductive molecules act on undifferentiated mesenchymal cells to initiate the endochondral cascade, ultimately resulting in the formation of bone. Studies of undifferentiated rat marrow stromal cells confirm that BMP-2 acts to directly stimulate osteoblast development, and that this stimulation is enhanced by the addition of dexamethasone (77). Others have shown that bone formation occurs in an orthotopic site when fresh marrow alone is added, but the rate and extent of healing is a function of the amount of marrow and the number of osteoprogenitor cells residing therein (26,49,129,144). An important set of experiments by Takagi and Urist (129) demonstrate that the addition of BMP is not effective at healing segmental defects when access to the medullary canal and the marrow stroma is prevented, thus indicating an absolute requirement for the cellular constituents of marrow in BMP-mediated bone repair. These results were bolstered by studies indicating that the implantation of fresh marrow along with BMP in a rat segmental gap model is more effective than either component implanted alone (74). One may conclude from all of the above that marrow-derived mesenchymal progenitors, or MSCs, are the target for endogenous osteoinductive molecules, such as BMPs, which are released during normal bone healing. It therefore follows that one must have an adequate supply of MSCs in order to respond to the normal (or exogenously supplied) signals of bone repair, or healing will be effete.

The histomorphometric data generated in this study provides a basis for comparison to other investigations. When fresh marrow from one femur equivalent is loaded on an HA/TCP implant, no significant difference in bone formation is observed when compared to implants which receive no cells. This is true for both time points in our study, and likely reflects an inadequate number of MSCs in the volume of marrow applied. Had we loaded the implants with

regeneration strategies is direct delivery of the cellular machinery required for bone formation. This approach would have an extraordinary advantage in settings where the number of endogenous progenitor cells is reduced, such as that which occurs in ageing, osteoporosis, or a variety of other pathologic conditions (33,72,82,118,128,135). Other investigators have pursued this logic by attempting to deliver more progenitor cells simply by concentrating the marrow, by crude fractionation and removal of red blood cells, or by cultivating the stromal cells *in vitro* (26,83,103,105,144). Now that techniques and conditions have been established which support the expansion of purified human MSCs in culture as much as one billion fold without a loss in osteogenic potential (13), analogous clinical protocols for regenerating human bone defects are not far away. It will be possible to further expedite the healing process by directing these culture-expanded MSCs *ex vivo* to enter the osteogenic lineage prior to implantation, thus decreasing the *in situ* interval between implantation and their biosynthetic activity as osteoblasts. Additional efforts are underway to develop cell delivery vehicles which will provide more flexibility to the surgeon, including materials which can be shaped to fit any type of defect. By combining a pharmacologic stimulus, such as BMP, with an even better delivery vehicle, we will be able to offer patients therapeutic options which have never before been available.

cultured under osteoinductive conditions to quantify aspects of osteoblastic differentiation.

Canine Femoral Gap Model

A unilateral segmental femoral defect model was developed for this study following IACUC approval. Under general anesthesia, thirty-six skeletally mature female purpose-bred hounds (20 kg) underwent resection of a 21 mm long osteoperiosteal segment from their mid-diaphysis. A 4.5 mm Synthes® 8-hole lengthening plate was contoured to the lateral aspect of the bone, and secured with bicortical screws. The defect was filled with one of three materials; 1) a cell-free HA/TCP cylinder, 2) an MSC-loaded HA/TCP cylinder, or 3) cancellous bone harvested from the iliac crest. HA/TCP implants were secured by placing two sutures around the implant and the plate. Animals received peri-operative antibiotics, and analgesics were administered for three days post-operatively.

Radiographic and Histologic Analyses

Standard radiographic images were obtained at pre-op, immediately post-op, and at 4 week intervals until termination of the study. All samples contained a radiodensity step wedge to provide a basis for comparing changes over time, and between dogs. Upon sacrifice, specimens were subjected to high resolution Faxitron radiography, and subsequently processed for biomechanical evaluation. Following torsion testing, undecalcified longitudinal sections will be processed for quantitative histomorphometry.

Biomechanical Testing

Sixteen weeks after implantation, animals were sacrificed for torsion testing of femurs. The fixation plate, screws, and adherent soft tissue were removed, and the metaphyses of the bones were embedded. The specimens will be externally rotated in a custom torsion test apparatus, failure load and stiffness recorded, and the data analyzed by one way ANOVA according to *post hoc* Student-Newman-Keuls tests.

with our observation that MSCs undergoing osteogenic differentiation secrete a paracrine factor(s) which is osteoinductive (63). The conspicuous lack of callus formation and periosteal reaction in the cell-free implants was an unexpected finding. Radiographic evidence suggests that MSC-mediated bone regeneration is faster than autograft throughout the study period. In addition to establishing a new standardized model for large animal bone repair, this study illustrates the feasibility of translating autologous stem cell therapy from the laboratory into the clinic.

Example 3

In vivo Bone Formation Using Human Mesenchymal Stem Cells

Although rat MSCs have been shown to synthesize structurally competent bone in an orthotopic site (68), human MSCs have only been shown to form bone *in vitro* (12,64) and in an ectopic implantation site in immunodeficient mice (55). Since fracture healing and bone repair depend on the ability to amass enough cells at the defect site to form a repair blastema, one therapeutic strategy is to directly administer the precursor cells to the site in need of repair. This approach is particularly attractive for patients who have fractures which are difficult to heal, or patients who have a decline in their MSC repository as a result of age (72,118), osteoporosis (128), or other metabolic derangement. With this in mind, the goal of the current study was to show that purified, culture-expanded human MSCs are capable of regenerating bone at the site of a clinically significant defect.

Materials and Methods

Human MSC Cultivation and Manipulation

Isolation and culture-expansion of human MSCs from a bone marrow aspirate obtained from a normal volunteer after informed consent was conducted as previously described (54,52). Following initial plating in Dulbecco's Modified Eagle's Medium (Sigma) containing 10% fetal bovine serum (BioCell) from a selected lot (80), non-adherent cells were removed on day 3 at the time of the first medium change, and fresh medium was replaced twice weekly thereafter. Adherent MSCs represent approximately 1 in 10^5 nucleated cells originally plated. When culture dishes became near-confluent, cells were detached and serially subcultured.

Quantitative Hist morphometry and Immunochemistry

Upon sacrifice at 4, 8, and 12 weeks, a minimum of 3 specimens of each type were processed for undecalcified histology following radiography. Longitudinal sections were cut, stained with Toluidine blue-O, and quantitative assessment of bone formation was performed using Leica Quantimet 500MC image analysis software as previously described (68). The data were analyzed by Student's t-test. Subcutaneously implanted samples were fixed in formalin, decalcified, embedded in paraffin, serially sectioned, and similarly stained. Limbs from one animal at each time point were also prepared for immunostaining by monoclonal antibody 6E2, which distinguishes human cells from rat cells (54). Undecalcified cryosections were incubated with 6E2 supernatant, or an irrelevant primary monoclonal antibody control (SB-1) (10), followed by FITC-conjugated goat anti-mouse IgG secondary antibody (GIBCO) diluted 1:500 in phosphate-buffered saline.

Biomechanical Testing

Twelve weeks after implantation, 7 experimental animals and 6 unoperated control animals were sacrificed for torsion testing of femurs as previously described (81). The fixation plate and adherent soft tissue were removed, and the metaphyses of the bones were embedded. The specimens were externally rotated in a custom torsion test apparatus, failure load and stiffness recorded, and the data analyzed by one way ANOVA with *post hoc* Student-Newman-Keuls tests.

Results

MSC Cultivation and Osteogenic Differentiation *In Vitro*

Human MSC cultures were established and, by 7 days, formed characteristic colonies on the surface of the culture dish. Primary colonies which were subcultivated on day 14 attached uniformly to the surface of new dishes, and were allowed to divide for another 7 days until they became ~85% confluent. Passaged cells demonstrated their characteristic spindle-shaped morphology (Fig. 6A), and uniformly divided resulting in an even distribution of MSCs throughout the plate. Cells derived from this first passage were used for preparing implants as previously described, and an aliquot was used to confirm their osteogenic potential *in vitro*.

which received MSC-loaded HA/TCP cylinders (Fig. 8B) versus cell-free cylinders (Fig. 8C). Increasing radiodensity, and obliteration of the apparent pore structure, was used as an indication of new bone formation within the core of the implant. Although integration of the implant, or union, was not generally observed by 4 weeks, the subsequent formation of a radiodense bone bridge between the implant and the host at 8 weeks completely masked the interface. By 8 weeks, the MSC-loaded implant contained considerable bone within the pores and was integrated with the host bone at the ends of the implant. At 12 weeks, union was complete and additional bone was evident in the pores. Callus formation along the fixation plate was observed in some samples, as was an occasional eccentric spicule of bone usually present along the medial aspect of the femur. Some specimens, both with and without cells, contained cracks within the core of the implant.

Immunocytochemical Evaluation

Immunocytochemical staining with antibody 6E2 demonstrates that, at 4 weeks, virtually all the cells within the pores of the implant were reactive on their surface and were, therefore, of human origin (Fig. 9A). Along the immediate periphery of the implant, the host rat cells were intermingled with the human donor cells, but as the distance away from the surface of the implant increased, the representation of donor cells precipitously declined. The presence of these peripheral cells which are not immunostained also serves as a negative control for this established antibody. The ceramic material itself, which appears black in the phase contrast micrograph (Fig. 9B), displays a high level of background fluorescence. The exquisite sensitivity of the 6E2 antigen:antibody interaction necessitated that we use unfixed frozen sections which, unfortunately, limited our ability to process these calcified tissue specimens for immunostaining. While we were able to obtain satisfactory cryosections of 4 week samples (shown here), we were unable to prepare sections from later samples which contained substantially more bone.

Histologic Evaluation

Analysis of the Toluidine blue-O-stained samples confirmed the observations made by radiography. Photomicrographs of representative sections of the implant groups recovered at 12 weeks are shown in Figure 9. Most of the pores of the implants loaded

Table 3

Bone Fill in HA/TCP Implants as a Percentage of Available Space

	Four Weeks	Eight Weeks	Twelve Weeks
Carrier Alone	1.89 ± 1.00	11.47 ± 7.08	29.51 ± 8.93
Carrier plus MSCs	1.95 ± 1.92	26.46 ± 3.60*	46.61 ± 14.83*

Table 3. Longitudinal sections through the segmental defect of athymic rats implanted with ceramic carriers, with and without human MSCs, were evaluated histomorphometrically for bone content . The results represent the mean ± SD of 3 experimental limbs of each group at 4 and 8 weeks, and 8 limbs of each group at 12 weeks. $*P<0.05$ compared to the carrier alone at each time point.

Bone present in the cell-free HA/TCP implants primarily represents the bony ingrowth from the cut ends of the host cortices. At 4 weeks and beyond, the MSC-loaded samples contained significantly more bone than the cell-free group, and the average bone fraction within the implant increased over time, reaching 26.5% and 46.6% by the 8 and 12 week time points, respectively. This increased bone fraction at 8 weeks is 2.3-fold higher than that measured in cell-free implants at the same time, and by 12 weeks, is over 23-fold higher than that observed in either condition at 4 weeks. The volume fraction of the HA/TCP carrier remained constant, and served as an internal control for histomorphometry.

Mechanical Testing

Twelve experimental and 11 intact femora from age and weight-matched control animals were tested in torsion 12 weeks after implantation. Two experimental limbs were not tested because they were extremely fragile. Gross inspection of the healed defects revealed a distal varus rotation deformation in most specimens. Table 4 summarizes the mechanical testing results in terms of torsional strength, stiffness, and total energy absorbed.

clinical application of autologous MSC-therapy for the treatment of orthopedic defects in man.

The progressive increase in radiodensity of the healing bone at 8 weeks parallels the histological observations of processed limbs. Immunocytochemistry proves that the cells associated with the ceramic at 4 weeks are of human origin, and that the cells surrounding the implant are from the host. At 8 weeks and beyond, bone is laid down by the donor MSCs and eventually resorbed and replaced by bone derived from host cells through the normal remodeling sequence (24,47). It is important to note that in the process of regenerating this osseous defect, bone formation occurs by a direct conversion of mesenchymal cells into osteoblasts rather than by an endochondral cascade. This observation is consistent with previous studies of osteogenesis in implants loaded with animal or human MSCs (32,70,54,68). As the regenerative process continues, the pores of the ceramic are filled with an increasing amount of bone, which is laid down upon the walls of the implant or existing bone, and oriented by the invading vasculature that provides a portal for the entry and establishment of new marrow islands containing hematopoietic elements and host-derived MSCs.

The rate of bone regeneration is lower than that observed in euthymic rats implanted with syngeneic MSCs (68), suggesting that immunocompromised rats are not the ideal hosts to assess the bone-forming potential of human MSCs. This may be due in part to the xenogeneic nature of the implant and the increased natural killer cell activity, which may be a compensatory mechanism for the animal to cope with its deficient T-cell-mediated immunity (123). Nevertheless, a significantly higher amount of bone was formed in the defect which received MSCs compared to those limbs receiving the carrier only. The extent of host-implant union was greater in the MSC-loaded implants, which likely reflects the combined contributions of implanted MSCs and host-derived cells.

The ability of human MSCs to regenerate bone in this experimental model compares favorably with other investigations testing implants such as demineralized bone matrix, bone marrow, purified or recombinant bone morphogenic proteins (BMP), allograft, ceramics, fibermetals and gene-activated matrices (129,34,147). In addition to forming a substantial amount of histologically normal bone, the biomechanical data

Example 4

Effect of C ating on the Osteogenic Response of MSC-Loaded HA/TCP Cubes

This experiment was performed in an attempt to establish that uncoated HA/TCP cubes are equivalent to fibronectin- or autologous serum-coated HA/TCP cubes in supporting MSC-mediated osteogenesis.

Materials & Methods

Standard HA/TCP cubes coated with either fibronectin, 1% autologous serum, 10% autologous serum or those left uncoated, were loaded with MSCs and implanted subcutaneously into athymic mice. The cubes were retrieved six weeks post-implantation and inspected for the level of osteogenesis by decalcified histological methods. The experiments were done with multiple human and canine donors, and were performed in duplicate mice.

Results & Conclusion

MSC-loaded cubes from all treatment groups showed a significant amount of bone formation at six weeks. The coating of HA/TCP cubes with either fibronectin or serum had no effect on the level of MSC-mediated osteogenesis within the cube. As expected, the cell-free control HA/TCP cubes did not have osteogenesis. Based on the above results, we conclude that uncoated HA/TCP is a viable carrier for the delivery of MSCs to effect bone repair/augmentation.

bone as a "bone graft expander" significantly reduced autograft performance ($p < 0.01$)(58). Interestingly, successful fusions resulting from the CC composite had similar mechanical properties to fusions resulting from autograft, indicating that residual unresorbed ceramic granules had no evident adverse effect on the material properties of the fusion mass.

We have evaluated bone marrow processing as a means of enhancing graft performance in a spinal fusion study, using the Collagen Corporation collagen/ceramic composite as a delivery system for a purified matrix protein, Osteoinductive Factor (OIF). This matrix was mixed 50:50 with either autogenous cancellous bone (AB), freshly aspirated bone marrow (ABM), or fresh bone marrow nucleated cells which had been concentrated ten fold by centrifugation and buffy coat isolation (BMC). Unfortunately, as the study was completed, it was discovered that OIF was not an active cytokine. Earlier preparations of OIF had been inductive due to contamination with BMPs. As a result, the overall fusion rate was low. However, there was a strong trend for improved results from marrow concentration when ABM and BMC are compared ($p=0.06$, Logistic Regression Model for Clustered Data). Unfortunately, our ability to answer this important question was limited by the poor baseline performance of the matrix. This highlights the importance of selecting an effective and reliable matrix for evaluation of bone marrow grafts in this project. The incidence of union scores from 0 to 4 for each material in this experiment are presented in Table 5.

possible combinations of the three materials in three sites, are prepared at the beginning of each experiment and placed in an envelope. Site assignments are then made intraoperatively by blind drawing after site preparation is complete. Internal fixation is applied to each segment using plates placed on either side of the spinous processes. No external immobilization is used. All animals are euthanized twelve weeks post-operatively lateral faxitron radiographs of each excised spine are obtained to assess the integrity of fixation. After removal of plates, high resolution CT images are acquired of all segments from L1-L6 in each spine. Individual fusion segments are mechanically tested to failure and physically evaluated for union. Comparison between materials is made based on union score, quantitative image analysis of CT data, and the mechanical properties observed for each fusion.

Three weeks prior to spinal fusion each animal undergoes aspiration of bone marrow from the left iliac crest using sterile techniques under short acting IV sedation. These samples will then be transported to isolate and proliferate mesenchymal stem cells.

On the day of surgery, each animal in the study undergoes spinal fusion at three separate levels. Mesenchymal stem cells are provided, and fresh bone marrow will be harvested again, this time from the right iliac crest. Each material composite is then prepared intraoperatively. Following preparation of the surgical bed and application of internal fixation, a 2 cc volume of each material is grafted at one level in each animal. In this way each animal has one fusion site for each of the materials. The materials are distributed according to a randomization protocol to prevent surgical bias and insure uniform distribution of materials by site.

Initial Bone Marrow Aspiration and MSC Preparation

Following sterile skin preparation, a small (3 mm) stab incision is made using a #11 blade. A Lee-Lok bone marrow aspiration needle (Lee-Lok Inc., Minneapolis, MN), is advanced into the bone cavity. The obturator is removed. A 2 cc volume of bone marrow is then aspirated promptly into a 10 cc syringe containing 1 cc of heparinized saline-(1000 units/ml). The syringe is detached and inverted several times to insure-mixing. Subsequent aspirates are taken using identical technique through the same skin incision and the same cortical window, but redirecting the needle tip to

adjoining laminar surfaces under continuous saline irrigation. Entry into the neural canal is avoided. When site preparation is complete, a saline soaked gauze is placed in each fusion site, the wound is covered; and an unscrubbed assistant blindly selects a card containing the material/site assignment for that animal.

Porous hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic material, supplied from Zimmer Inc. as a 60/40 combination, is lightly crushed and sieved to select for a particle size ranging from 1.0 to 2.5 mm in diameter. Following sterilization of the granules, 15 million MSCs are incubated with the 1 cc of ceramic for 3 hours at 37 degrees Celsius with agitation every 30 minutes. As noted above, implant groups consist of 1) ceramic granules combined with autologous MSCs, 2) ceramic granules combined with autologous MSCs and fresh bone marrow obtained by aspiration, and 3) ceramic granules combined with fresh bone marrow alone.

Following preparation of all graft materials, materials are carefully placed in their appropriate sites. Approximately 1 cc of each graft is used to fill the area of the excised facets and the interlaminar space. The remainder of each graft is layered over the dorsal surface of the adjoining lamina.

Fixation at each site is then applied. A 1 mm burr is used to place a hole in the central region of the distal spinous process at each site. 316L stainless steel plates (0.125" x 0.4" x 1.4") are placed on either side of the adjoining spinous processes and fixed to the caudal spinous process using a stainless steel bolt and nut (size 2-56, 0.5" long). Four symmetrical holes in each plate allow selection among three potential fixation lengths to accommodate variation in the interspinous distance at individual sites (0.75", 0.90", and 1.05"). The fixation to the cranial spinous process at each level is accomplished by drilling through the appropriate hole in the plates to create a hole in the spinous process and by passing a second bolt and nut. Both bolts are tightened firmly without crushing the spinous processes. A second locking nut is then applied at each site to prevent loosening. The spinal wound and the autograft donor site is then closed using O-Dexon Plus sutures in deep fascia, interrupted subcutaneous sutures of 2-0 Dexon Plus, followed by staples in the skin.

cleaned of soft tissue, taking care not to damage the fusion mass and to prevent dehydration of the specimen. Segments are then potted in Orthodontic Resin (Meer Dental Supply, Cleveland, OH) and frozen in double sealed plastic bags at -20°C.

Quantitative CT Image Analysis of Fusion Sites

Quantitative assessment of the fusion mass is performed using helical X-ray Computed Tomography (CT) and automated 3-dimensional image processing techniques. Quantitative measures at each fusion site includes: 1) fusion mass volume, 2) area measured at the mid-disk cross-sectional plane, and 3) mineralization density of the fusion mass.

Scanning is done on a Somatome Plus 40 CT scanner (Siemens Medical Systems). The specimen is placed with the cranial-caudal axis perpendicular to the direction of table travel. In this orientation the intervertebral spaces are oriented perpendicular to the scan plane. This reduces the chance of missing features of nonunions occurring in the transaxial plane and allows the midline of the intervertebral space to be identified more accurately. Scans will be done at 120 kVp, 210 mA, 1 sec helical mode, 2 mm collimation, and table speed 2 mm/sec, for 30 secs. This will produce images with 2 mm section thickness and 1 mm^2 pixel area. Images are then reconstructed using the bone algorithm and an image-to-image overlap of 1 mm. This provides the highest possible spatial resolution available in three dimensions (i.e. a voxel size of 1 mm^3). A Siemens bone mineral density phantom is placed beneath each specimen to provide a reference for the quantitation of bone mineralization density.

Bone and soft tissue CT values are easily differentiable (CT bone - 150-1000, CT soft tissue - 0 ~ 20). Therefore, three-dimensional segmentation of the reconstructed volume data sets is performed using a basic automated threshold algorithm in each two dimensional slice followed by a connectivity algorithm between slices. Once the three-dimensional data set has been segmented, the volume of the fusion mass is measured in a specified region of interest, defined by anatomic fiducial markers. The user selects two points which define the dorsal most points of the left and right neural foramen at the level of interest and one level above and below the site of interest. The user also identifies the midpoint of the intervertebral disk. The interactive tools for

degree of union is scored from zero to four based on a regional grid system (Figure 11). A score of four is defined as complete fusion of both facet joints and the entire lamina. One half point is added for union in either half of each facet joint or any of four quadrants of the adjoining laminar surfaces. Therefore, scores of 0, 1, 2, 3 and 4 represent union of roughly 0, 25, 50, 75 and 100 percent of the cross-sectional area of the grafted volume, respectively. Scoring is done by consensus of two observers who examine the specimens,together and are blinded with respect to the material grafted at each site.

Statistical Analysis of Spinal Fusion Union Score and CT Data

Union score and CT generated data are analyzed to determine whether outcome differs statistically due to site and/or material effects. The data set is considered "incomplete" since only one material can be tested at each site within an animal. Therefore, both site and material are repeated factors but with missing observations. To accommodate the missing data profile of the experimental design as well as the possible correlation of observations within the same animal, a regression model for repeated measures data is used. This modeling strategy uses the Generalized Estimating Equation (GEE) approach described by Zeger *et al.* (150) and Ou *et al.* (108). Since union scores for a given dog are potentially not independent of each other, a working covariance structure of independence and a robust variance estimate are used in fitting the model. The covariance structure cannot be ignored without impacting inferences on the regression coefficients for site and material effects. T-tests using the robust variance estimates are used for specific comparisons between materials and sites as described by Paik (112). Union score remains the primary outcome parameter, however, distributional properties of the CT data will eventually dictate the statistical modeling strategy.

Statistical Analysis of Spinal Fusion Mechanical Testing Data

Mechanical parameters are strongly influenced by union score. Therefore, mechanical testing is used in this model as a secondary outcome parameter, primarily as a means to compare the material properties of bone formed in complete fusions (Large cross sectional area-Union Score 3.5 to 4.0) induced by different materials. Selection of complete unions allows comparison of fusions having similar moments of inertia. The complexity of bony geometry in partial fusions and wide local variation in the quality of

Example 6

Bone Defect Repair Using Bone Marrow in an Absorbable Collagen-Containing Sponge

The objectives of this study were to demonstrate efficacy of bone marrow and/or mesenchymal stem cells (MSCs) in healing clinically significant bone defects in an established animal model.

Materials & Methods

In the study, Fisher 344 rats (Charles River Laboratories, Wilmington, MA) of approximately 325 grams in weight were used. A bilateral femoral gap 8 mm in length was created in each femur. This length is approximately towards the diameter of the mid-diaphysis of the femur. An internal fixation plate was applied with four Kirschner wires. The groups for comparison were separately treated with one of the following:

(1) Gelfoam® sterile sponge (Upjohn - Kalamazoo, MI);

(2) Gelfoam® sterile powder;

(3) Peripheral blood clot;

(4) Peripheral blood clot plus marrow derived from four bones;

(5) Gelfoam® sponge containing marrow derived from four bones.

(6) Gelfoam® sponge plus varying amounts of marrow from one bone down to one-half of one bone in the presence and absence of fresh peripheral blood to provide clot.

In this animal system, fresh marrow from four bones yields approximately 150 million cells while fresh marrow from one-half of one bone yields approximately 20 million nucleated cells. Each group consisted of a minimum of three animals, all of which were sacrificed six weeks post-operatively to obtain the desired end-points. Some animals received high-resolution Faxitron radiographs at an intermediate point three weeks after implantation. At the six-week time point when all animals were sacrificed, the limbs were removed, radiographed, and prepared for undecalcified histological evaluation.

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What Is Claimed Is:

1. A method for augmenting bone formation in an individual in need thereof by administering to said individual isolated human mesenchymal stem cells with a medium which supports the differentiation of such stem cells into the osteogenic lineage to an extent sufficient to generate bone formation therefrom.

2. The method of claim 1 wherein the medium is a porous ceramic or resorbable biopolymer.

3. The method of claim 2 wherein the ceramic is selected from the group consisting of hydroxyapatite, β-tricalcium phosphate and combinations thereof.

4. The method of claim 2 wherein the ceramic is in particulate form.

5. The method of claim 2 where the ceramic is a structurally stable, three dimensional implant.

6. The method of claim 5 where the structurally stable, three dimensional implant is a cube, cylinder, block or in the shape of an anatomical form.

7. The method of claim 2 wherein the resorbable biopolymer is selected from the group consisting of a gelatin, collagen and cellulose.

8. The method of claim 7 wherein the medium is a powder, sponge, strip, film, gel or web or a structurally stable, three dimensional implant in the form of a cube, cylinder or block or in the shape of an anatomical form.

9. The method of claim 7 wherein the gelatin is a bovine skin-derived gelatin.

10. The method of claim 1 which further comprises administering to said individual at least one bioactive factor which induces or accelerates the differentiation of such mesenchymal stem cells into the osteogenic lineage.

19. The method of claim 10 wherein the bioactive factor is a synthetic glucocorticoid.

20. The method of claim 11 wherein the bioactive factor is a synthetic glucocorticoid.

21. The method of claim 12 wherein the bioactive factor is a synthetic glucocorticoid.

22. The method of claim 19 wherein the synthetic glucocorticoid is dexamethasone.

23. The method of claim 20 wherein the synthetic glucocorticoid is dexamethasone.

24. The method of claim 21 wherein the synthetic glucocorticoid is dexamethasone.

25. The method of claim 10 wherein the bioactive factor is a bone morphogenic protein.

26. The method of claim 11 wherein the bioactive factor is a bone morphogenic protein.

27. The method of claim 12 wherein the bioactive factor is a bone morphogenic protein.

28. The method of claim 25 wherein the bone morphogenic protein is in a liquid or semi-solid carrier suitable for intramuscular, intravenous, intramedullary or intra-articular injection.

38. The composition of claim 37 wherein the resorbable biopolymer is in particulate form.

39. The composition of claim 37 wherein the resorbable biopolymer is a sponge, strip, film, gel or web or a structurally stable, three dimensional implant.

40. A method for augmenting bone formation in an individual in need thereof which comprises administering to said individual thereof a bone formation augmenting amount of the composition of claim 34.

41. A method for augmenting bone formation in an individual in need thereof which comprises administering to said individual thereof a bone formation augmenting amount of the composition of claim 35.

42. A method for augmenting bone formation in an individual in need thereof which comprises administering to said individual thereof a bone formation augmenting amount of the composition of claim 36.

43. A method for augmenting bone formation in an individual in need thereof which comprises administering to said individual thereof a bone formation augmenting amount of the composition of claim 37.

44. A method for augmenting bone formation in an individual in need thereof which comprises administering to said individual thereof a bone formation augmenting amount of the composition of claim 38.

45. A method for augmenting bone formation in an individual in need thereof which comprises administering to said individual thereof a bone formation augmenting amount of the composition of claim 39.

FIG. 3A

FIG. 3B

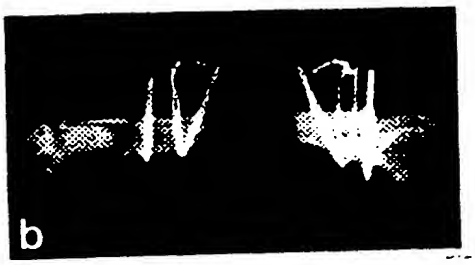

FIG. 3C

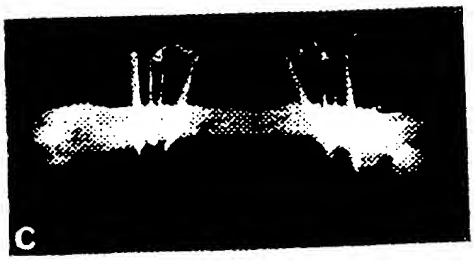

FIG. 3D

FIG. 3E

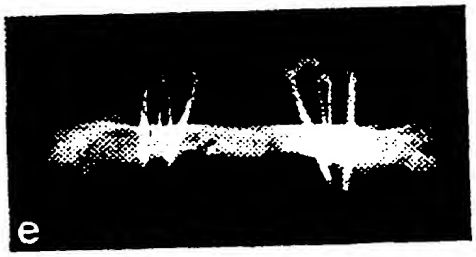

FIG. 3F

FIG. 3G

FIG. 3H

FIG. 4D

FIG. 4E

FIG. 4F

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